Impact of vancomycin on sarA-mediated biofilm formation: role in persistent endovascular infections due to methicillin-resistant staphylococcus aureus

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Abstract: Background. Staphylococcus aureus is the most common cause of endovascular infections. The staphylococcal accessory regulator A locus (sarA) is a major virulence determinant that may potentially impact methicillin-resistant S. aureus (MRSA) persistence in such infections via its influence on biofilm formation. Methods. Two healthcare-associated MRSA isolates from patients with persistent bacteremia and 2 prototypical community-acquired MRSA strains, as well as their respective isogenic sarA mutants, were studied for in vitro biofilm formation, fibronectin-binding capacity, autolysis, and protease and nuclease activities. These assays were done in the presence or absence of sub–minimum inhibitory concentrations (MICs) of vancomycin. In addition, these strain pairs were compared for intrinsic virulence and responses to vancomycin therapy in experimental infective endocarditis, a prototypical biofilm model. Results. All sarA mutants displayed significantly reduced biofilm formation and binding to fibronectin but increased protease production in vitro, compared with their respective parental strains. Interestingly, exposure to sub-MICs of vancomycin significantly promoted biofilm formation and fibronectin-binding in parental strains but not in sarA mutants. In addition, all sarA mutants became exquisitely susceptible to vancomycin therapy, compared with their respective parental strains, in the infective endocarditis model. Conclusions. These observations suggest that sarA activation is important in persistent MRSA endovascular infection, potentially in the setting of biofilm formation.

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Impact of Vancomycin on *sarA*-Mediated Biofilm Formation: Role in Persistent Endovascular Infections due to Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT

Background. *Staphylococcus aureus* is the most common cause of endovascular infections. The staphylococcal accessory regulator A, *sarA,* is a major virulence determinant that may potentially impact MRSA persistence in such infections via its influence upon biofilm formation.

Methods. Two healthcare-associated MRSA isolates from patients with persistent bacteremia and two prototypical community-acquired MRSA strains, and their respective isogenic *sarA* mutants were studied for *in vitro*: **i**) biofilm formation; **ii**) fibronectin-binding capacity; **iii**) autolysis; and **iv**) protease and nuclease activities. These assays were done in the presence or absence of sub-MIC vancomycin. In addition, these strain-pairs were compared for intrinsic virulence and responses to vancomycin therapy in experimental infective endocarditis (IE), a prototypical biofilm model.

Results. All *sarA* mutants displayed significantly reduced biofilm formation and binding to fibronectin, but increased protease production *in vitro* vs. their respective parental strains. Interestingly, sub-MIC exposure to vancomycin significantly promoted biofilm formation and fibronectin-binding in parental strains, but not in *sarA* mutants. In addition, all *sarA* mutants became exquisitely sensitive to vancomycin therapy vs. their respective parental strains in the IE model.

Conclusions. These observations suggest that *sarA* activation is important in persistent MRSA endovascular infection, potentially in the setting of biofilm formation.

INTRODUCTION

Staphylococcus aureus is the most common cause of endovascular infections, including infective endocarditis (IE) syndromes which are associated with high morbidity and mortality [1]. The increase in prevalence of methicillin-resistant *S. aureus* (MRSA) strains in such infections, and the relatively high rates of vancomycin clinical failures in these syndromes caused by MRSA strains whose MICs fall within the susceptible range (MICs ≤ 2 μg/ml) have further complicated the management of these patients [2]. In addition, MRSA is a predominant cause of healthcare-associated (HA) infections [3]. However, life-threatening infections caused by community-acquired (CA)-MRSA in otherwise healthy people outside of health care settings have recently reached epidemic proportions globally [4].

The pathogenesis of *S. aureus* is complex and involves the coordinate expression of multiple gene products including surface adhesins, exoproteins and toxins. Of note, many staphylococcal virulence factors are controlled by a major regulatory locus called staphylococcal accessory regulator A, *sarA* [5]. The *sarA* locus consists of three overlapping transcripts, driven by three distinct promoters: P1, P3 and P2. These DNA-binding proteins bind to AT-rich inverted repeat or palindromic sequences on respective target promoters to control the expression of multiple genes [5]. Importantly, laboratory-derived *sarA* mutants have exhibited diminished virulence in infection models, including IE, osteomyelitis and arthritis [6, 7].

Biofilm formation plays a critical role in the development of MRSA endovascular infections [8]. Treatment of these infections has become challenging since organisms within the

biofilm are significantly more resistant to antimicrobial agents, as well as to clearance by the host defense system [9]. In addition, recent studies suggest that sub-lethal doses of selected antibiotics (e.g., vancomycin) can induce biofilm formation in MRSA strains [10]. In this regard, SarA is an important positive regulator of biofilm development, in part due to its repressive activity on protease production [11-13]. For the first time to our knowledge, the current study examines the impact of vancomycin on *sarA*-mediated biofilm formation in MRSA *in vitro,* as well as *in vivo* in terms of intrinsic virulence and treatment outcomes in endovascular infections.

MATERIALS AND METHODS

MRSA Strains. Two HA-MRSA isolates, selected from a multi-national clinical trial, were from patients demonstrating ≥ 14 days of positive MRSA blood cultures despite receiving antibiotics to which the isolates were susceptible *in vitro* (**Table 1**) [14, 15]. In addition, two prototypical CA-MRSA strains, MW2 (USA400, reported to cause fatal infections in children [16]) and JE2 (LAC, USA300 derivative that was cured of its three plasmids, obtained from NARSA [Network on Antimicrobial Resistance in *Staphylococcus aureus*]) were also chosen for this study. A site-specific mutant was obtained by transducing the *sarA*::Tn917LTV1 mutation from strain ALC637 (Newman, *sarA*:Tn917LTV1) [17] into strain 324-136 using phage 80α (erythromycin^r). A *sarA* deletion in strains JE2 and 300-169 was achieved by transducing the *sarA*::*kan* mutation from ALC2543 (COL with a *sarA::kan* mutation) [18] using phage 85 (kanamycin^r). The study MRSA strains were routinely grown in tryptic soy broth (TSB) or on TS agar (TSA).

Minimum inhibitory concentration (MIC) determination. MICs for vancomycin were determined by standard broth microdilution method as recommended by the Clinical and Laboratory Standards Institute [19].

***In vitro* time-kill curve of vancomycin.** The bactericidal activities of vancomycin (range, 1- to -5x MICs) were compared between the parental strains and their respective *sarA* mutants by use of killing curve analyses [20].

Population analyses assay. Vancomycin population analyses were performed by standard protocols [21]. The range of vancomycin concentrations tested was 0.125 to 16 μg/ml to encompass sublethal-to-lethal drug levels using an initial inoculum of ~10^9 CFU/ml. Population analysis curves were compared between each strain-pair [8].

Biofilm formation. Biofilm formation of the study MRSA strains was performed under static conditions [8, 22]. Briefly, MRSA cells from fresh culture plates were adjusted to a density of 0.5 McFarland standard, and diluted 1:100 into brain heart infusion (BHI) supplemented with 0.5% glucose; 200 μl of this suspension was transferred to 96-well tissue culture plates and incubated for 18 h at 37°C. After incubation, the wells were washed, air dried, stained with Safranin (0.1% in distilled water). The adhering dye was dissolved in 30% acetic acid, and absorption was measured at OD_{490nm} to quantify biofilm formation [8, 22].

Numerous investigations have shown that sub-MIC levels of some antibiotics can induce bacterial biofilm formation [8, 10, 23]. In this regard, we have recently reported that sub-MIC

vancomycin exposure can promote MRSA biofilm formation, a process that may have clinical significance [8]. Therefore, the above static biofilm formation assay was performed in parallel with exposure of 0.5x MIC of vancomycin.

Biofilm stability. Bacterial biofilms consist of carbohydrates, proteins and extracellular DNA (eDNA) in variable proportions [9, 22]. Distinct biofilm chemical compositions are felt to contribute functionally and structurally to the organization of biofilm [9]. Therefore, differences in the stability of MRSA biofilm as a function of specific biochemical components were separately assessed in the presence of carbohydrate, protein, or DNA dispersal agents [22]. The supernatants of 18h-old biofilms generated by the study MRSA strains were replaced by fresh medium supplemented with either: **i**) 10 mM sodium metaperiodate; **ii**) 100 µg/ml proteinase K; or **iii**) 140 U/ml RNase-free DNase I to assess relative carbohydrate, protein and DNA content, respectively [22]. All incubations were for 2h at 37°C. Media without the above supplements served as respective controls. After treatment, the biofilms were quantified as described above.

Autolysis assay. Standard Triton X-100-induced autolysis was performed as described [18]. In addition, it has been reported that increased staphylococcal autolysis mediated by sub-MIC vancomycin exposure can augment biofilm formation [10]. Thus, the MRSA strains were also examined for vancomycin-induced lysis. In brief, overnight cultured strains were adjusted to an OD_{580nm} of 0.7, washed, exposed to 50 mM Tris-HCl (pH 7.2) containing either 0.05% Triton X-100 or 0.5x MIC of vancomycin, and incubated at 30°C with agitation (200 rpm). Staphylococcal lysis was measured during 24 h incubation by determining the changes in OD_{580nm}.

Adherence to fibronectin. For these assays, six-well tissue culture plates were coated with purified human fibronectin (50 μg/ml) for 18 h, then treated with 3% bovine serum albumin for 3 h to prevent nonspecific adhesion [24]. Overnight cultured MRSA cells grown in the presence and absence of 0.5x MIC vancomycin were added to the plates (5x 10^3 CFU) and incubated for 1 h [24]. Wells were then washed, and TSA were added to each well, incubated at 37°C for overnight. Adherence was expressed as the percentage (± SD) of the initial inoculum bound.

Protease and nuclease activity. MRSA extracellular proteases and nucleases are essential modulators of biofilms, being involved in the degradation of the biofilm matrix; they may also play a critical role in the biofilm-deficient phenotype in the *sarA* mutants [9, 25, 26]. Therefore, we tested overall extracellular protease and nuclease production of the study strains using 1% caseinate agar (for proteolysis activity) and DNase agar (for nuclease activity) plates in the presence or absence of 0.5x MIC of vancomycin [26, 27].

Rabbit model of MRSA Infective endocarditis (IE). A well-characterized rabbit model of catheter-induced aortic valve IE with intravenous (iv) injection of ~10^5 CFU of each MRSA strain was used [15]. Rabbits were maintained in accordance with the American Association for Accreditation of Laboratory Animal Care criteria. The Animal Research Committee (IACUC) of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center approved all animal study protocols. At 24 h after infection, animals were randomized to receive: **i**) no therapy [controls]; or ii) vancomycin at 15 mg/kg or 7.5 mg/kg, iv twice daily for three days for HA- and CA-MRSA strain sets, respectively. At 24 h after the last vancomycin dose, animals were sacrificed, and their cardiac vegetations, kidneys, and spleen were removed and quantitatively cultured [15]. MRSA counts in target tissue are given as the mean $\log_{10}$ CFU/g of

tissue (± SD).

Assessment of *sarA* transcriptions *in vitro* and within cardiac vegetations *in vivo* by quantitative real-time PCR (qRT-PCR). For *sarA* transcriptions *in vitro*, RNA was isolated from cultures that were grown for 24 h with/without 0.5x MIC of VAN exposure in order to compare to data generated *in vivo* within cardiac vegetation samples collected 24 h post-infection (see below) [15]. Briefly, two micrograms of DNase-treated RNA was transcribed into cDNA [15]. qRT-PCR was carried out using an ABI Prism 7000 instrument (Applied Biosystems) and the SYBR green PCR master kit (Applied Biosystems). The primers used to amplify *sarA* were qRT-sarA-F (5'-TCTTGTTAATGCACAACAACGTAA-3') and qRT-sarA-R (5'-TGTTTGCTTCAGTGATTCGTTT-3'). *gyrB* was used to normalize for transcript quantification [15]. Relative quantification was calculated by the $\Delta\Delta C_T$ method [28]. PCR experiments were performed using two biological replicates, each tested in triplicate.

For quantifying *sarA* transcripts within cardiac vegetations, catheterized animals were infected with each parental strain as described above. At 24 h post-infection, animals were randomized to receive: **i**) no therapy; or ii) vancomycin as described above. Vancomycin was given for one day only (to assure adequate numbers of MRSA cells within cardiac vegetations for RNA extractions). At 24 h after the last vancomycin dose, animals were sacrificed, and their cardiac vegetations were quick-frozen in liquid nitrogen. Total RNA was isolated using Tri reagent (Ambion) according to the manufacturer's instructions, including a ¼-in. ceramic sphere in the cell disruption step [15]. Samples were then processed as described above. Quantitative RT-PCR analysis for *sarA* expression was performed on at least three different animals for each group.

RESULTS

Vancomycin MICs.

Vancomycin MICs were 0.5 μg/ml for the two HA-MRSA strains, and 1.0 and 2.0 μg/ml for CA-MRSA strains, MW2 and JE2, respectively. In addition, no differences were detected in vancomycin MICs between the parental and their respective *sarA* mutant strains.

In vitro time-kill curve of vancomycin.

The results of *in vitro* time-kill curve of vancomycin are shown in **Fig. 1**. No significant differences in vancomycin bactericidal effects were observed between parental and *sarA* mutant strains. In addition, no strains exhibited *in vitro* tolerance to vancomycin at 5x MIC exposure levels (**Fig. 1**).

Population analyses. None of the study isolates revealed vancomycin hetero-resistant subpopulations (hVISA; data not shown). In addition, there were no population curve shifts in comparing the parent and *sarA* mutant strains within each strain-pair.

Biofilm formation under static conditions. Consistent with previous reports [11, 12], all four *sarA* mutation were defective in biofilm formation as compared to their respective parental strains (**Fig. 2A**). Interestingly, sub-lethal (0.5x) MIC levels of vancomycin significantly promoted biofilm formation in both HA-MRSA parental strains and in the CA-MRSA parental strain, JE2 as compared to their respective parental controls without vancomycin exposure (**Fig. 2B**). However, vancomycin exposure did not significantly change the biofilm formation profile

in the MW2 parental strain (**Fig. 2B**). Importantly, sub-lethal vancomycin exposure did not induce biofilm formation in any of the *sarA* mutants.

Biofilm stability. Treatment with sodium metaperiodate, proteinase K or DNase I led to a similar significant reduction of biofilm formation in the two HA-MRSA parental strains as compared to respective controls without exposure to any dispersal agent (**Supplementary Fig. S1**). However, treatment with sodium metaperiodate did not cause a significant reduction of biofilm formation in two CA-MRSA parental strains vs. their respective controls without sodium metaperiodate exposure, suggesting that biofilm formed by these CA-MRSA strains might have relatively lower carbohydrate content (vs. protein and DNA; **Fig. S1**). For all the *sarA* mutants, treatment with sodium metaperiodate had little effect on the biofilm stability, whereas there was significant biofilm dispersion by treatment with proteinase K and DNase in two of the four *sarA* mutants, suggesting these two *sarA* mutant strains have less carbohydrate content as compared with protein and DNA content (**Fig. S1**).

Cell Autolysis. No differences in autolysis profiles were observed between parental and *sarA* mutant strains in the presence of either Triton X-100 or 0.5x MIC vancomycin (data not shown).

Adherence to fibronectin: As anticipated from prior studies [24, 29], inactivation of *sarA* led to significantly reduced fibronectin binding vs. their respective parental strain ($p < 0.05$, **Fig. 3A**). Interestingly, in the presence of 0.5x MIC vancomycin, two HA-MRSA parental strains showed significantly increased binding to fibronectin vs. their respective controls, whereas the two CA-MRSA strains exhibited decreased fibronectin binding (although this did not reach statistical

significance) (**Fig. 3B**). For all the *sarA* mutants, fibronectin binding did not increase with vancomycin exposure (**Fig. 3B**).

Protease and nuclease activity. In accordance with previous studies showing that *sarA* negatively regulates protease and nuclease gene expression [12], the *sarA* mutants demonstrated increases in proteases and nuclease activity in all study strains, except for strain JE2 (in which nuclease activity did not change; **Fig. 4**). Of note, although sub-MIC levels of vancomycin had a positive impact on biofilm formation as documented above, there were no differences in either protease or nuclease production induced by vancomycin (**Fig. 4**).

Experimental infective endocarditis (IE). At the 10^5 CFU challenge inoculum, three of the four *sarA* mutants (excluding JE2) exhibited reduced virulence as compared to their parental strains in terms of MRSA densities achieved within cardiac vegetations (but not kidneys or spleen; $p < 0.05$, **Fig. 5**). Of note, vancomycin treatment resulted in dramatic and significant reductions of MRSA counts in all target tissues in animals infected with the *sarA* mutant strains (3.8-6.6 $\log_{10}$ CFU/g. tissue reduction; $p < 0.0005$ vs. untreated *sarA* mutant controls; **Fig. 5**). In addition, vancomycin treatment resulted in ~80% culture-negativity in all target tissues in animals infected with *sarA* mutants in 300-169 and 324-136 strain backgrounds; also 18% and 100% of organ cultures were sterile in animals infected with *sarA* mutant in MW2 and JE2 backgrounds; respectively. In contrast, rabbits infected with three of the four parental strains (two HA-MRSA and MW2, but not JE2) did not respond to vancomycin treatment, with residual target tissue MRSA densities in vancomycin-treated animals being similar to those in their respective untreated control groups (**Fig. 5**).

***sarA* transcriptions *in vitro* and within cardiac vegetations.** With 0.5x MIC VAN exposure *in vitro*, all parental strains exhibited significantly higher *sarA* transcript levels than their respective controls in the absence of VAN exposure (**Fig. 6A**). For *sarA* expression within cardiac vegetations, higher *sarA* expression profiles were observed in the VAN treatment groups vs. their respective controls without VAN therapy (**Fig. 6B**). Interestingly, higher *sarA* expression was detected in HA-MRSA vs. CA-MRSA parental strains without VAN treatment. However, these differences did not reach statistic significance (**Fig. 6B**).

DISCUSSION

Biofilm formation is considered to be a major virulence factor in many *S. aureus* syndromes, including IE [9, 25]. Biofilm formation not only facilitates bacterial colonization of host tissues, but also fosters resistance to bacterial clearance mediated by antimicrobial agents and by host immune responses [9, 25]. In addition, biofilms can serve as chronic foci of infection for metastatic spread of bacteria and release of toxins into the bloodstream, resulting in significant morbidity and mortality [9]. Therefore, biofilm formation has become a key target for the development of novel therapeutic tools against these life-threatening infections. In this study, we investigated the modulation of a central regulatory element that controls the production of many *S. aureus* virulence factors (including biofilm), *sarA*, as means of enhancing antimicrobial efficacy in a prototypical MRSA biofilm infection model (IE).

In the current investigation, consistent with other reports, we observed that *sarA* mutants formed significantly less biofilm *in vitro* in all study strains as compared to their respective parental strains [11, 12]. It is well-known that *sarA* has a global effect on many *S.□aureus*

virulence genes that seem to play a role in biofilm formation (e.g., multiple extracellular proteases, nuclease and fibronectin-binding proteins [FnBPs]) [30]. For instance, recent studies and our own current data show that the overall production of extracellular proteases is increased in *sarA* mutants, which may contribute to the reduced biofilm phenotype in such strains [26, 31, 32]. In addition, there is growing evidence that the release of eDNA from bacterial cells undergoing autolysis during programmed cell death contributes to biofilm development [33, 34]. Therefore, an increased production of extracellular nuclease may contribute to the biofilm-deficient phenotype of *sarA* mutants [34, 35]. Moreover, nuclease (*nuc*) mutants form a very thick biofilm containing increased levels of matrix-associated eDNA [33]. In this regard, in the current study, three of our four *sarA* mutants exhibited increased extracellular nuclease production, again correlating with reduced biofilm formation. Taken together, these results suggest that the regulatory role of *sarA* in repressing the production of extracellular enzymes (including proteases and nucleases) is one key factor leading to biofilm formation and stability [26].

S. aureus biofilm matrices consist of proteins, DNA, and polysaccharide (also called the polysaccharide intercellular adhesion [PIA] or poly-N-acetylglucosamine [PNAG] material) [9, 22]. Recently, it has become evident that the presence of PIA is not essential for biofilm development in many MRSA strains [29]. Interestingly, in these PIA-independent biofilms, FnBPs appear to "substitute" for PIA in driving biofilm formation [29]. In addition, this FnBP-mediated biofilm seems to be particularly frequent among highly virulent MRSA isolates, suggesting the importance of PIA-independent biofilm formation in such strains [29]. SarA is a positive regulator of FnBP production and subsequent biofilm formation in two complementary

ways: **i**) by enhancement of *fnb* gene expression; and **ii**) by repression of extracellular proteases which normally cleave and remodel surface adhesins such as FnBP [12, 13]. Thus, we tested the fibronectin-binding capacities of our study strains. Our results, in agreement with other studies [24, 36], found that all *sarA* mutants had significantly decreased capacity to bind to fibronectin. This reduced fibronectin-binding phenotype was correlated with increased protease production observed in *sarA* mutants, ostensibly contributing to reduced bacterial colonization at the early steps of endovascular infection.

Several groups have shown that sub-MIC levels of selected antibiotics, including vancomycin, can promote *S. aureus* biofilm formation *in vitro* [10, 37, 38]. In the current studies, we applied this concept to characterize the impact of sub-MIC levels of vancomycin on *sarA*-mediated biofilm formation in a persistent endovascular infection model due to HA- and CA-MRSA strains. Vancomycin was chosen in this investigation because it remains a clinical mainstay as an anti-MRSA agent. We confirmed that sub-MIC vancomycin significantly promoted biofilm formation in three of four MRSA parental strains (excluding MW2). In contrast, all *sarA* mutants demonstrated reduced biofilm formation in the presence or absence of sub-MIC vancomycin *in vitro*. In addition, we demonstrated that sub-MIC vancomycin exposure significantly increased *sarA* gene expression in all study parental strains studied *in vitro* vsersus their respective controls without vancomycin exposure. This outcome was mirrored *in vivo*, as vancomycin treatment resulted a trend towards higher MRSA *sarA* expression within IE cardiac vegetations vs. respective controls without vancomycin therapy. These phenotypic and genotypic differences in the impact of sub-MIC vancomycin on biofilm formation and *sarA* expression paralleled *in vivo* outcomes. Thus, animals infected with parental MRSA strains were resistant to vancomycin treatment, while animals infected with *sarA* mutants were exquisitely susceptible to

vancomycin therapy in the IE model.

We recognize that additional mechanisms likely contribute to the differences in vancomycin-associated outcomes *in vivo* as noted above. For example, vancomycin has limited and slow penetration into biofilms [39, 40]. Therefore, one intriguing possibility is that *sarA* mutant strains form significantly thinner and/or less well-structured biofilms *in vivo* as compared to their respective parental strains. In turn, this latter phenotype may conceivably allow greater penetration of vancomycin into such defective biofilms. Further, Hsu *et al*. [10] reported that sub-lethal doses of vancomycin can induce more robust biofilm formation through an enhanced autolysis- and eDNA-dependent release in *S. aureus*. In contrast to their findings, however, the present studies were unable to detect any impact of sub-MIC vancomycin on either autolysis or nuclease or protease production, suggesting these latter mechanisms were not in-play in the current study strains.

Lastly, we noted that mutations in *sarA* had a definable, albeit modest effect on innate MRSA virulence, being observed only with in cardiac vegetations. This impact of *sarA* on IE pathogenesis has been seen in prior studies in this model using other *S. aureus* strains [6, 24, 41]. Of major importance, vancomycin therapy in the experimental IE model was able to divulge the ability of *sarA* to blunt antimicrobial efficacies, presumably via a biofilm-dependent pathway. Interestingly, these *in vivo* results were observed despite similar vancomycin MICs and *in vitro* vancomycin killing kinetics between *sarA* mutants and their respective parental strains. These results suggest that *sarA* plays a key role in endovascular infections, especially in terms of antimicrobial therapy responsiveness, likely, at least in part, due to the decreased production of

extracellular protease, resulting in augmented biofilm formation.

In summary, for the first time in our knowledge, the present results reveal the importance of *sarA* in two critical aspects of *S. aureus* pathogenesis in endovascular infections caused by clinical isolates: biofilm formation and target tissue persistence. Although the mechanism(s) of these phenomena are not entirely defined, these data support the notion that suppression of SarA has therapeutic potential in the important context of biofilm-associated infections due to HA- and CA-MRSA.

TABLES

Table 1. ***S. aureus*** **strains used in this study**

Strains	Relevant characteristics	Reference
ALC637	Newman *sarA*:Tn917LTV1, erm^R	[17]
ALC2543	COL *sarA*::*kan*	[18]
300-169	HA-MRSA (persistent bacteremia patient)	[15]
300-169 Δ*sarA*	300-169 *sarA::kan*	This study
324-136	HA-MRSA (persistent bacteremia patient)	[15]
324-136 Δ*sarA*	324-136 *sarA::Tn917LTV1*	This study
MW2	CA-MRSA, USA 400	[18]
ALC5415	MW2 *sarA::kan*	[18]
JE2	CA-MRSA, LAC, USA 300	[42]
JE2 Δ*sarA*	JE2 *sarA::kan*	This study

FIGURE LEGEND

Figure 1. *In vitro* time-kill curves of vancomycin against HA-MRSA (Panel A: 300-169 and 324-136) and CA-MRSA (Panel B: MW2 and JE2) strain sets. ■, Control; ◇, 1x MIC of vancomycin; △, 2x MIC of vancomycin; ┌,5x MIC of vancomycin.

Figure 2. Impact of *sarA* on biofilm formation. Panel A, biofilm formation of the wild types and their isogenic *sarA* mutants was quantified using a microtitler plate assay by solubilizing the safranin in 30% acetic acid and absorption was measured at 490nm. Results are shown as the mean of the A_{490nm} ± SD from three independent experiments, each of which was done in triplicate. **P*<0.005; ***P*<0.0005 the *sarA* mutants vs. their respective parental strain. Panel B, effect of 0.5x MIC of vancomycin (VAN) treatment on biofilm formation. Biofilm formation without VAN exposure was defined as being A_{490nm} 1.0, and represented by the dashed line. ■, parental; □, *sarA* mutant; * $P < 0.05$ vs. control without VAN exposure.

Figure 3. Adherence of the study MRSA strain sets to immobilized human fibronectin in the absence (Panel A) and presence (Panel B) of 0.5x MIC vancomycin (VAN). Controls without VAN exposure set up 100% adherence to fibronectin (panel B). ■, parental; □, *sarA* mutant; * $P < 0.05$ vs. their respective parental strain (Panel A) or vs. controls without VAN exposure (Panel B).

Figure 4. Production of extracellular protease and nuclease by the study strain sets in the absence and presence of 0.5x MIC vancomycin (VAN). Results were assessed after overnight

incubation of the study strains on caseinate and DNase agar plates for protease and nuclease, respectively. P: parental strain; S: *sarA* mutant strain.

Figure 5. MRSA densities in target tissues in the IE model with and without vancomycin (VAN) treatment (15 mg/kg and 7.5 mg/kg iv, bid for 3 days for HA-MRSA and CA-MRSA strains, respectively). Panel A: HA-MRSA strain sets; and Panel B: CA-MRSA strain sets. Each dot represents one rabbit. Horizontal black bars indicate means of observations.

Figure 6. Expression of *sarA* in all study MRSA parental strain with/without 0.5x MIC VAN exposure at 24 h *in vitro* (A) and within infected cardiac vegetations with/without VAN therapy in the IE model at 24 h post-infection (B) by qRT-PCR. Relative transcript levels of *sarA* represent the mean (+ SD) of two biological replicates *in vitro* and of at least three animals per group *in vivo* (fold changes versus *gyrB*). * indicates $P < 0.05$; and ** indicates $P < 0.005$ compared with their respective controls without VAN exposure *in vitro*.

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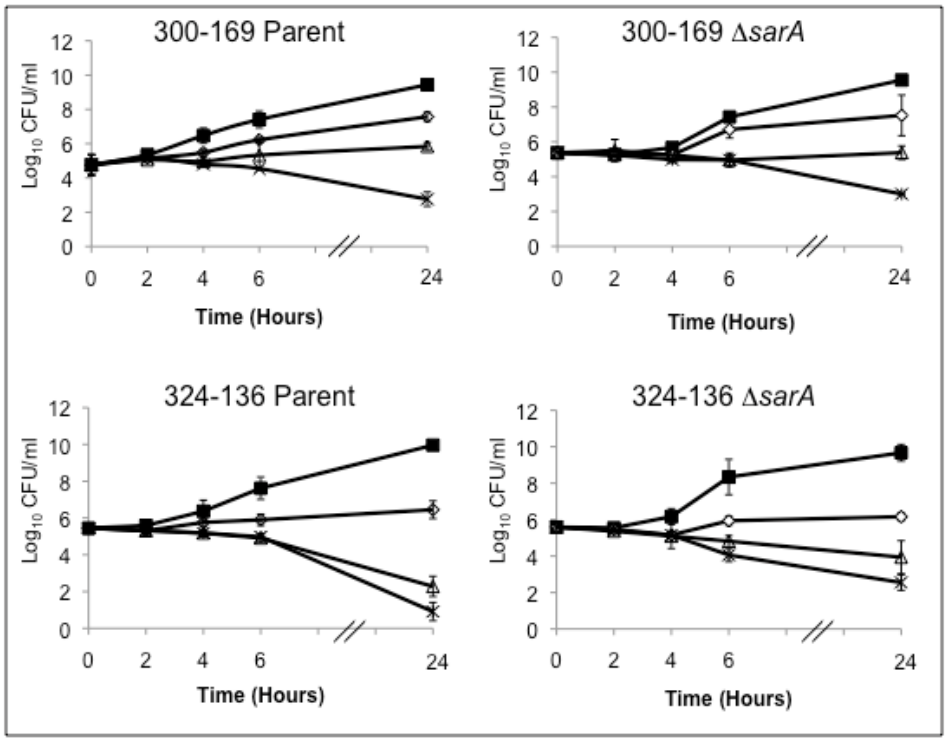
Figure 1



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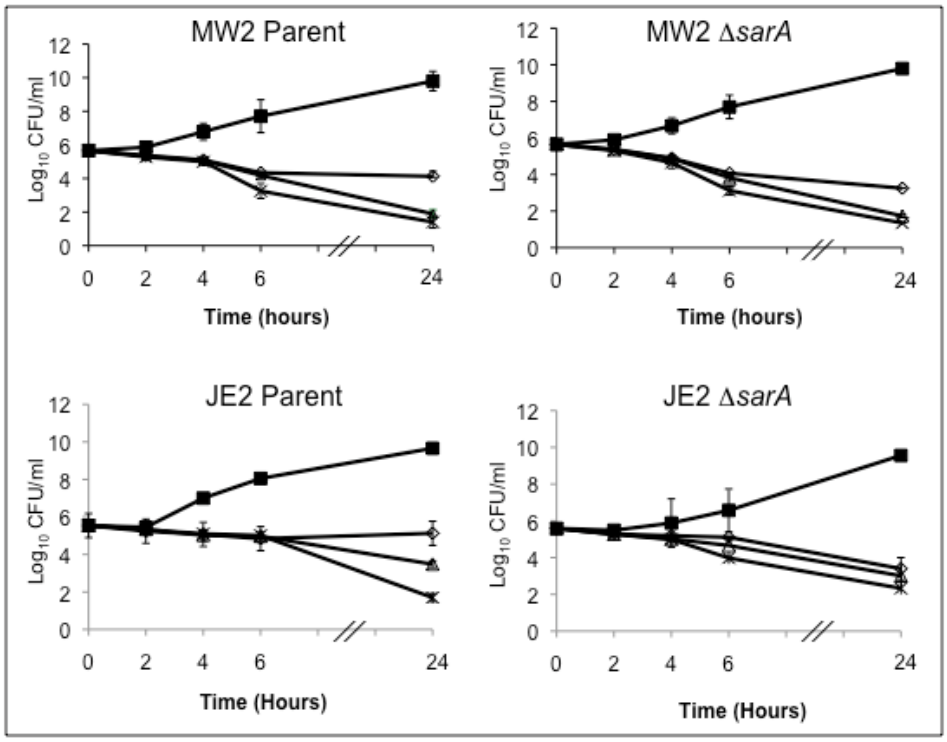
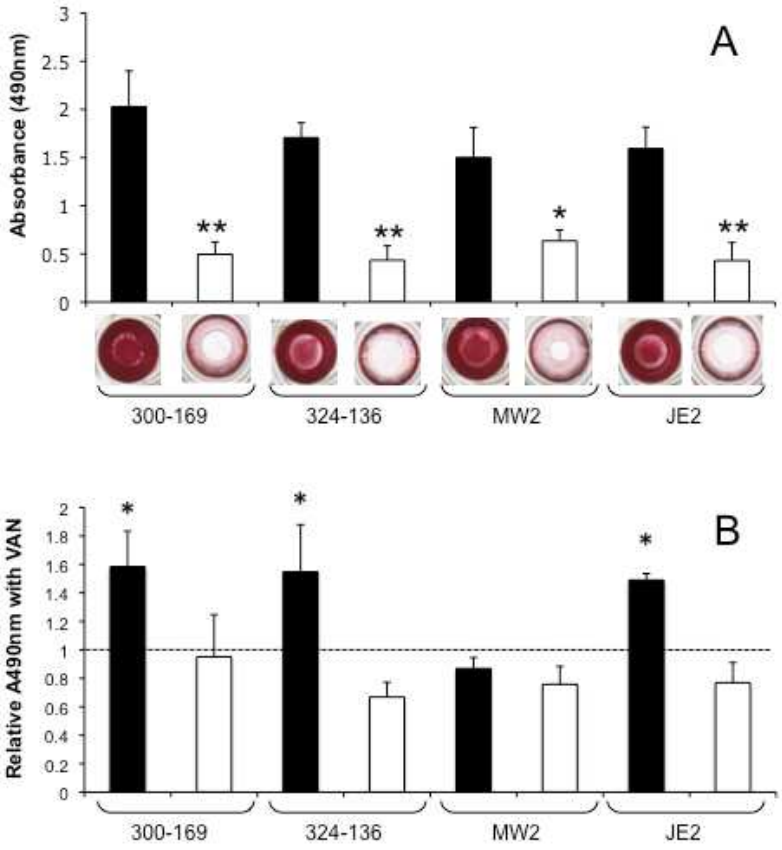


Figure 2



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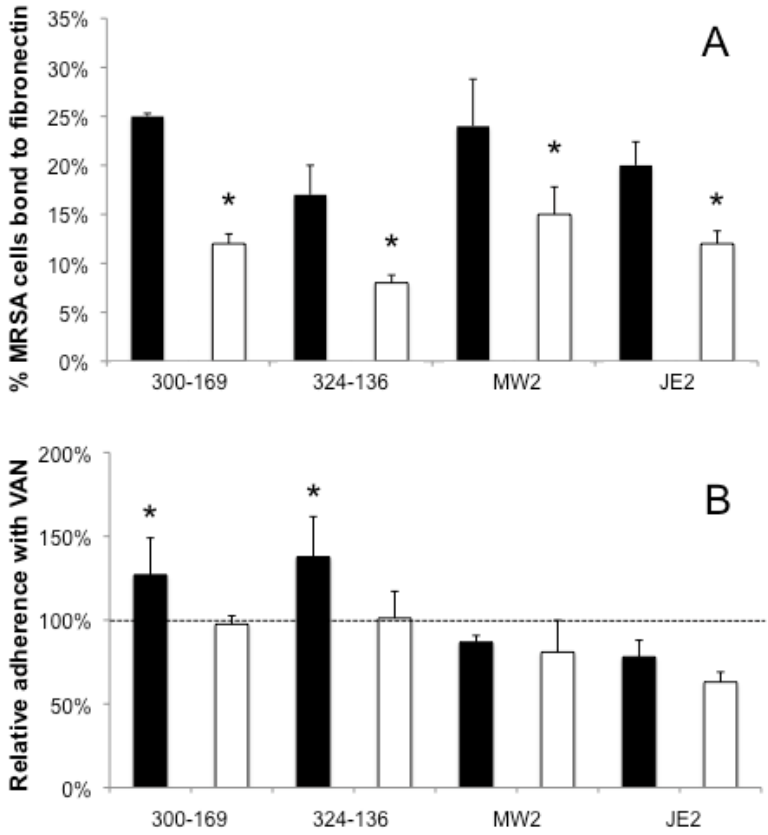

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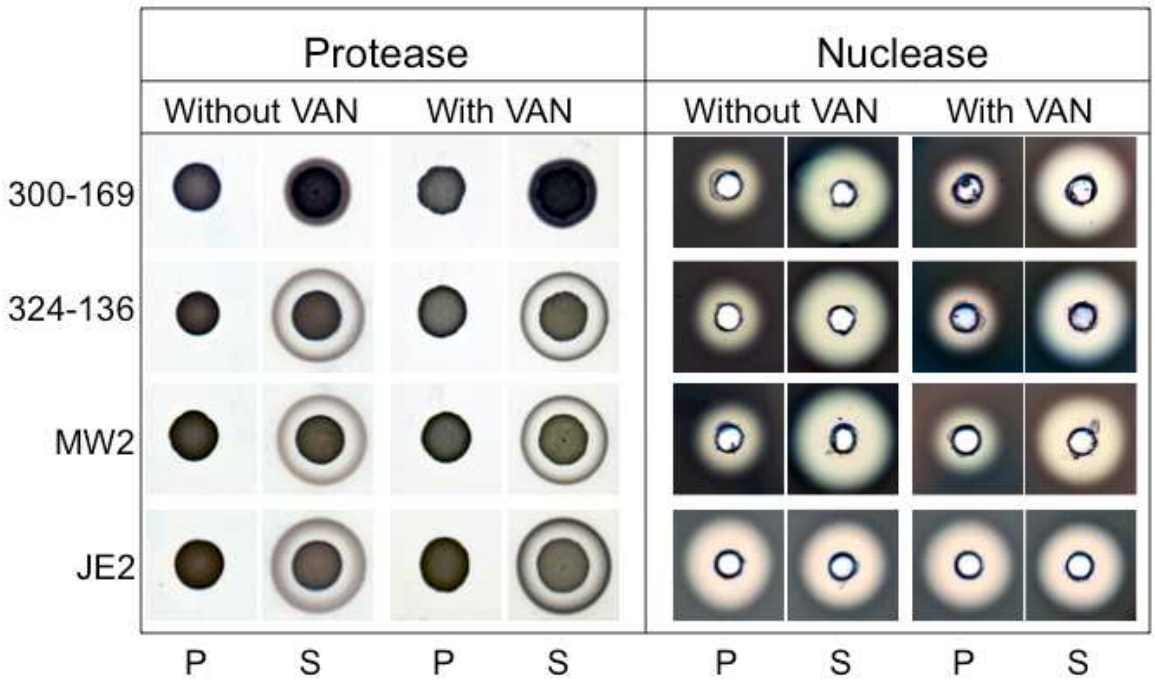
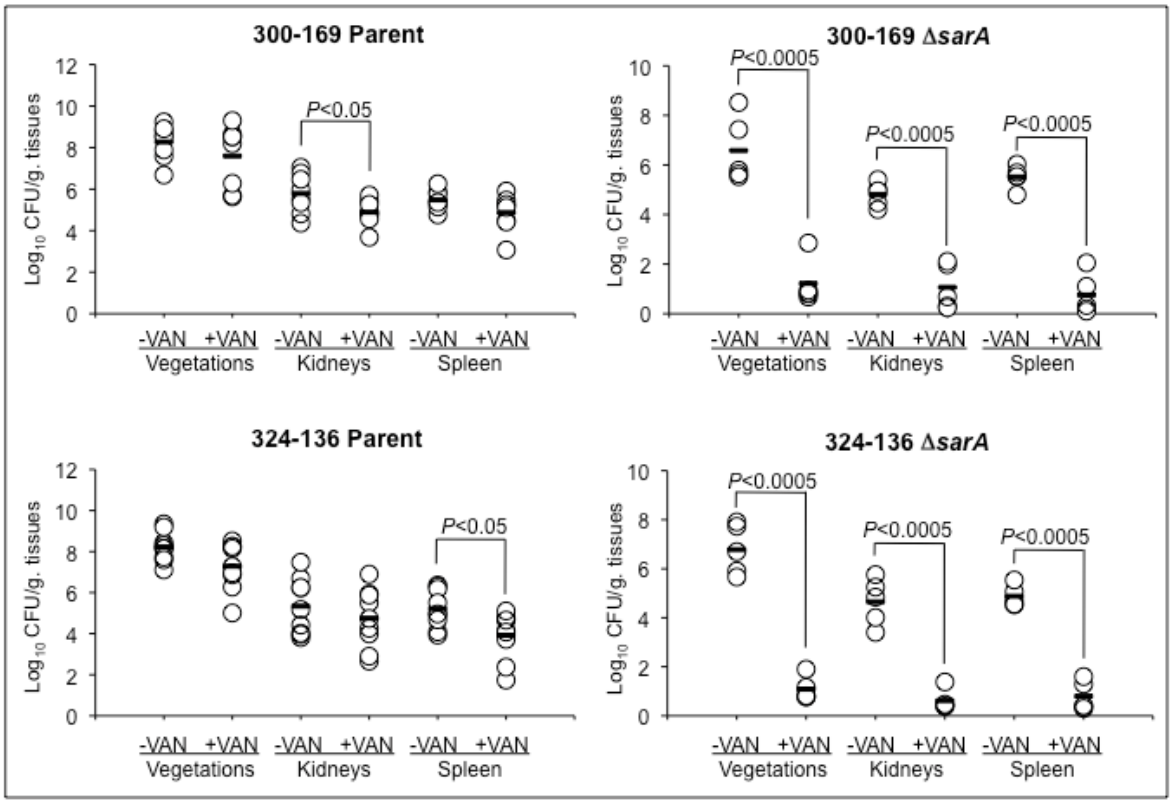


Figure 5

A



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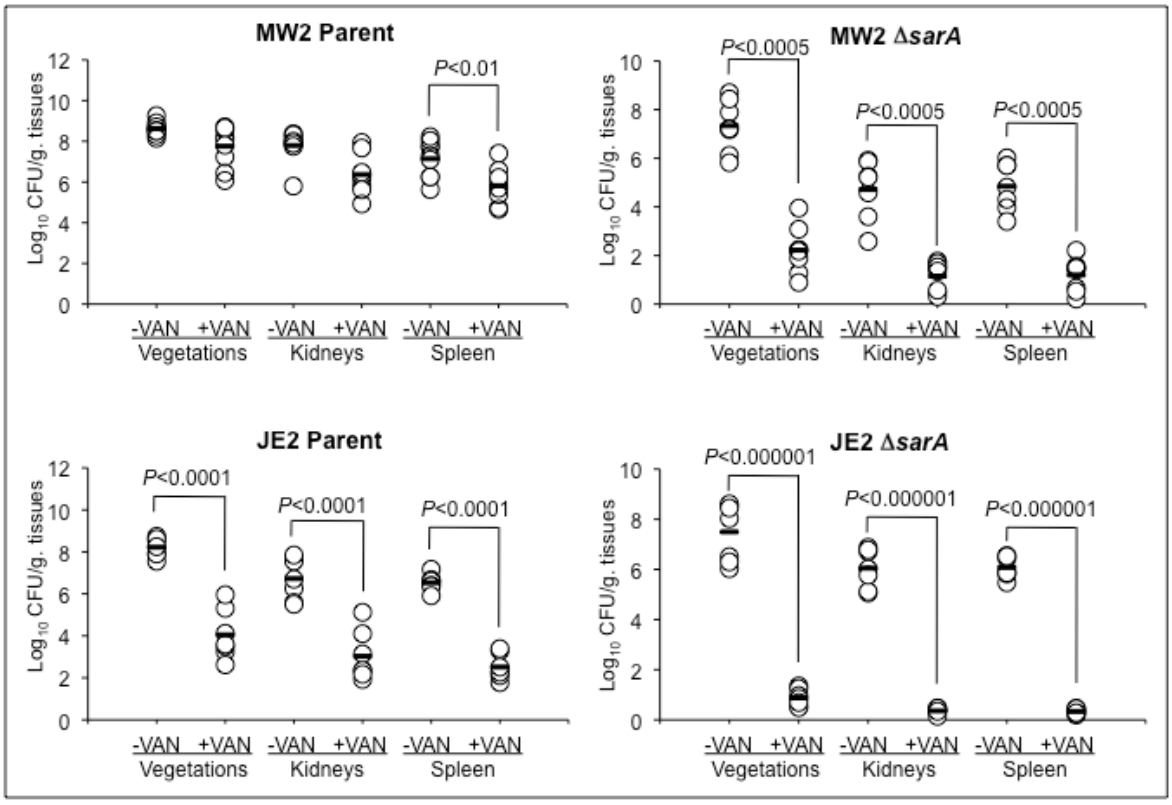

Figure 6